Differential contribution of the two major polygalacturonases from *Penicillium digitatum* to virulence towards citrus fruit

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Abstract

The fungus *Pencillium digitatum* is the causal agent of the citrus green mould, the major postharvest diseases of citrus fruit. Lesions on the surface of infected fruits first appear as soft areas around the inoculation site, due to maceration of fruit. The macerating activity has been associated with pectinases secreted by the fungus during infection. In order to evaluate the contribution to virulence and macerating activity of the two major polygalacturonases (PGs) secreted by *P. digitatum*, we have obtained and characterized mutants lacking either *pg1* or *pg2*, the genes encoding PG1 and PG2, respectively. Disease incidence of deletants in either gene was not different from that of the parental strain or ectopic transformants. However, disease progressed more slowly in deletants, especially in those lacking the *pg2* gene. The lesions originated by the Δ*pg2* deletants were not as soft and the pH was not as acid as those originated by either the wild type strain or the ectopic transformants. Total PG activity in the macerated tissue was also lower in fruits infected with the Δ*pg2* deletants. Interestingly, the macerated tissue of oranges infected with Δ*pg2* deletants showed around 50% reduction in galacturonic acid content with respect to lesions caused by any other strain.

1. Introduction

Green mould rot, caused by *Penicillium digitatum*, is the most common postharvest disease affecting citrus fruit in Spain (Tuset, 1987) and all production areas characterized by low summer rainfall (Eckert and Eaks, 1989). This pathogen may invade the fruit during the preharvest period through injuries occurred in the field, or/and in the packinghouses during storage and shelf-life periods. *P. digitatum* is a specialist pathogen that under natural conditions infects citrus fruit uniquely, although previous works demonstrated that it can infect overripe apple tissues (Buron-Moles et al., 2012; Vilanova et al., 2012; Vilanova et al., 2014). The use of synthetic fungicides has been the standard procedure to control this pathogen (Harding, 1972). However, these chemical treatments have several disadvantages, such as the persistence of the residues on the treated fruit, increase of the pathogen-resistant strains, as well as health and environmental problems (Bus, 1992). New approaches for designing new and safer control strategies would benefit from the knowledge of the molecular mechanisms underlying the pathogenesis of *P. digitatum*.

P. digitatum is a necrotrophic wound pathogen that requires pre-existing injured fruit peel to penetrate the plant tissue (Kavanagh and Wood, 1967). Necrotrophs kill host cells by means of toxic molecules, which can be either host-specific, as tentoxin, or nonhost-specific toxins, as AM toxin, and lytic enzymes. However, the ultimate purpose of a necrotroph is not to kill its host, but to discompose the plant tissue and utilize the host-derived nutrients for its own growth (Zhang and van Kan, 2013). During infection, necrotrophic plant pathogens macerate the host tissue by secreting significant amounts of carbohydrate-active enzymes (CAZYmes) that contribute to the degradation of plant cell wall polymers to obtain the nutrients required for its development (Zhao et al., 2013). Among these CAZYmes special attention has been paid to those involved in pectin degradation. Pectin is the collective name for a complex of polysaccharides that constitute the

major carbohydrate type in the middle lamella (Jayani et al., 2005, Caffall and Mohnen, 2009). The most abundant type of pectin is homogalacturonan, a linear polymer of α-1,4-linked D-galacturonic acid, which can be modified by acetylation and methyl-esterification. Other pectins include rhamnogalacturonan I and II, and xylogalacturonan. Enzymes involved in the degradation of the pectin backbone include polygalacturonases (PGs), pectate and pectin lyases (PLs), rhamnogalacturonases and rhamnogalacturonase lyases (recently reviewed by Ramoni and Seiboth (2016)). Depending upon the pattern of action (random or terminal) polygalacturonases (PGs) are termed as endo- or exo-enzymes, respectively. Endo- PGs are widely distributed among fungi, bacteria and many types of yeast whereas, in contrast, exo-PGs occur less frequently (Jayani et al., 2005).

PGs play a critical role in pectin degradation by fungal pathogens and they hydrolyse the polygalacturonic acid chain across the oxygen bridge (Jayani et al., 2005). PG activity has been detected in decayed tissue and has been implicated as a virulence factor in several soft rot diseases (Reignault et al., 2008). In some pathogens, the disruption of PG genes reduced virulence, which suggests that this enzyme is a significant virulence factor in several plant-infecting fungi (Scott-Craig et al., 1990; Shieh et al., 1997). However, in several other cases, disruption of cell wall-degrading enzymes caused only partial or no reduction in pathogenicity, suggesting that not all enzymes produced by the pathogen are required for pathogenicity (Scott-Craig et al., 1990). However, few studies on *P. digitatum's* cell walls degrading enzymes (CWDEs) encoding genes as virulence factors have been conducted so far (López-Pérez et al., 2015; Zhang et al., 2013a; Zhang et al., 2013b). Zhang and collaborators (2013b) have shown that a *P. digitatum* mutant lacking the polygalacturonase PG2 was able to infect citrus fruits, although it was less virulent than

85 the parental strain. A similar phenotype has been described for *P. digitatum* mutants lacking the
86 pectin lyase PL1, which showed reduced virulence (López-Pérez et al., 2015).

87 In a recent work, the importance of CWDEs in the virulence of *P. digitatum* was highlighted
88 because they constituted the second most abundant group of genes in a library containing up-
89 regulated fungal genes during the infection of oranges (López-Pérez et al., 2015). The genome of
90 *P. digitatum* is enriched in two families involved in pectin degradation, when compared to *P.*
91 *chrysogenum*, a closely related but not pathogenic species. Thus, *P. digitatum* possesses eight
92 polygalacturonases and rhamnnogalacturonases belonging to family GH28 and three pectin
93 methylesterases belonging to family CE8 (Marcet-Houben et al., 2012).

94 In order to clarify the role of *P. digitatum*'s PGs in pathogenicity, different strategies including
95 physiological, biochemical and molecular investigations should be performed. In this work, we
96 have compared the role in virulence of the two major PGs in *P. digitatum* by obtaining and
97 characterizing in the same genetic background knockout mutants for the genes *pg1* and *pg2*, to
98 provide evidence that these genes play a different role during pathogenesis on orange fruit.

99 **2. Materials and Methods**

100 **2.1. Fruits**

101 'Valencia' orange fruits (*Citrus sinensis* L. Osbeck) were harvested from a commercial orchard in
102 Tortosa (Catalonia, Spain) and processed the same day. Fruits were selected for uniform size,
103 without physical injuries or apparent infections. Once the fruit arrived at the laboratory, they were
104 surface-disinfected with a 10 % commercial bleach solution for 1 min, rinsed with tap water and
105 allowed to dry at room temperature. Colour index, firmness, soluble solids and acidity were
106 determined as quality parameters following standard procedures (Vilanova et al., 2013).

2.2. Fungal strains and culture conditions

Conidial suspensions from *Penicillium digitatum* Sacc. isolate Pd1 (CECT20795; (Marcet-Houben et al., 2012) were prepared by adding 5 mL of sterile water with 0.01 % (w/v) Tween-80 over the surface of seven- to 10-day-old cultures grown on potato dextrose agar medium (PDA; 200 mL boiled potato extract, 20 g dextrose, 20 g agar and 800 mL water) and rubbing the surface of the agar with a sterile glass rod. Conidia were counted in a haemocytometer and diluted to 10^6 conidia mL^{-1} inoculum concentration.

2.3. Construction of *P. digitatum pg1* and *pg2* disruption plasmids

P. digitatum Pd1 *pg1* and *pg2* genes correspond to NCBI gene entries PDIP_64460 and PDIP_19910, respectively. Further annotation of PDIP_64460 was required to match GenBank AB015286 sequence. Plasmids were constructed following the procedures described by López-Pérez et al. (2015). DNA fragments of 1.5-1.7 kb in length located upstream and downstream of both genes were amplified by PCR from *P. digitatum* Pd1 genomic DNA using primers pairs pg1-O1/pg1-O2 and pg2-O1/pg2-O2 (see Table 1 for primers sequences) for the upstream regions of *pg1* and *pg2*, respectively, and pg1-A3/pg1-A4 and pg2-A3/pg2-A4 for the downstream regions. The amplified upstream, containing the first exon of the gene, and downstream regions from each gene were cloned flanking the hygromycin B resistance cassette in the vector pRF-HU2 (Frandsen et al., 2008), which was previously digested with *Pac*I and *Nt.Bbv*CI, following the USER friendly cloning technique (New Englands Biolabs, Beverly,MA, USA) to generate plasmids pRFDPG1 and pRFDPG2, respectively. An aliquot of each plasmid was used to transform *E. coli* DH5 quimio-competent cells. Kanamycin resistant colonies were screened for proper fusion of both upstream and downstream gene flanking fragments by PCR with primers RF1-RF6 and RF2-RF5

and verified by DNA sequencing. Selected plasmids were electroporated into *Agrobacterium tumefaciens* AGL-1 electrocompetent cells.

2.4. *Agrobacterium tumefaciens*-mediated transformation of *Penicillium digitatum*

P. digitatum transformation was conducted as previously described (Marcet-Houben et al., 2012). Putative transformants were selected on PDA plates supplemented with hygromycin B (100 µg/ml) and cefotaxime (200 µg/ml). They were transferred to Eppendorf tubes containing PDA supplemented with hygromycin B (100 µg/ml) and cefotaxime (200 µg/ml) and incubated at 24 ºC until sporulation. Conidia were inoculated into liquid GPY medium (glucose 40 g/l; peptone 5 g/l; yeast extract 5 g/l) supplemented with hygromycin (100 µg/ml) and incubated at 24 ºC and 200 rpm for 2 days. DNA was extracted as described previously (López-Pérez et al., 2015). Insertion of the T-DNA in the transformants was verified by PCR with the primers HMBF1/HMBR1 (Table 1), which target the hygromycin B resistance gene. Integration by homologous recombination was analysed with primers pairs pg1-F7/pg1-R7 and pg2-F7/pg2-R7 for *pg1* and *pg2*, respectively. The absence of the targeted gene in the deletants was further verified using the primers pg1-F8/pg1-R8 and pg2-F8/pg2-R8 (Table 1) for *pg1* and *pg2*, respectively. Fig. 1A and 2A show a scheme with the relative position of the primers used in the characterization of the transformants.

The number of T-DNA insertions present in selected monosporic transformants was determined by qPCR following the procedure described by Crespo-Sempere et al. (2013) using the primer pairs pg1-F9/pg1-R9 and pg2-F9/pg2-R9, which are located in the PCR-amplified upstream regions of *pg1* and *pg2*, respectively. The *P. digitatum* gene encoding β-tubulin (GenBank accession number GU124566) was used as a reference for normalization employing primers betatubPDIG1/betatubPDIG2 (Table 1). DNA from the wild-type Pd1 strain was used as a control.

151 PCR reactions were performed using a LightCycler 480 Real-Time apparatus (Roche, Manheim,
152 Germany) and the LightCycler 480 SYBR Green I Master kit (Roche) following the manufacturer's
153 recommendations.

154 **2.5. Fruit infections**

155 Each orange was wounded by making two injuries with a nail (1 mm wide and 2 mm deep) in one
156 side of fruit and was then inoculated with 15 µL aqueous conidia suspension of *P. digitatum*
157 transformants or *P. digitatum* wild type strain. Oranges inoculated with sterile water were used as
158 control treatment. After inoculation, oranges were stored at 20 ºC and 85 % relative humidity (RH)
159 for four days. Decay incidence and severity were measured. Four replicates per treatment were
160 used and each replicate consisted of two inoculated wounds in five fruits.

161 **2.6. pH**

162 Mesocarp pH was determined by placing a micro-pH electrode directly into the wound (pH & Ion-
163 Meter GLP 22 + Model 5033 pH electrode, Crison Instruments SA, Barcelona, Spain). Four
164 replicates per treatment were used and each replicate consisted of two inoculated wounds in five
165 fruits.

166 **2.7. Firmness measurements**

167 Rot firmness was evaluated by measuring the maximal strength of compression of the infected
168 lesion area using a TA-TX2 Texture Analyzer (Stable Micro Systems Ltd., Surrey, England). The
169 resistance of the compression was measured using a cylinder probe with a round basis (P/0.75S)
170 using the following conditions: pre-test speed (2 mm/s), test speed (0.3 mm/s), post-test speed (5.0
171 mm/s) and contact distance (2 mm) and results were expressed as Newtons (N). Two replicates per
172 treatment were used and each replicate consisted of two inoculated wounds in five fruits.

2.8. Polygalacturonase activity assays

Two peel discs of 12 mm in diameter around the inoculation site and 4 mm deep were removed from 10 oranges using a cork borer. Twenty peel disks were so obtained, frozen immediately in liquid nitrogen, freeze-dried for 3 days, grounded to a fine powder in a coffee mill and stored at -80 ºC until further analysis as described below. Twenty discs pooled from ten fruits were considered one replicate. Three replicates per treatment were used.

For the extraction of polygalacturonase (exo-PG; EC 3.2.1.67 and endo-PG; EC 3.2.1.15) activity, 100 mg of freeze-dried peel tissue from each replicate was homogenised (10 %, w/v) in extraction buffer prepared according to Lohani et al. (2004). PG activity was determined on the crude extracts as referenced in Ortiz et al. (2011). One unit (U) of PG activity was defined as the liberation of 1 μmol of GalUA min^{-1} from citrus pectin (d.e. 70–75%), with galacturonic acid (GalUA) as a standard. Total protein content was determined with the Bradford (1976) method, with BSA as a standard. Results were given as specific activity over total protein (U mg-1 protein).

2.9. Analysis of organic acids

Two peel discs of 12 mm in diameter around the inoculation site and 4 mm deep were obtained in a similar manner to that used in the PG activity studies described above. Twenty discs from ten fruits were pooled and considered one replicate and four replicates per treatment were performed.

Malic, ascorbic, oxalic, citric and fumaric acids were extracted and quantified using high performance liquid chromatography (HPLC), and gluconic and galacturonic acids using ultra-high-performance liquid chromatography–mass spectrometry (UHPLC–MS) system (Waters, Milford, USA) following the conditions described by Vilanova et al. (2014). Results were expressed as mg g^{-1} dry weight (DW).

195 **2.10. Statistical analysis**

196 Data regarding incidence and severity of fruit decay, quality parameters, pH, firmness, PG activity
197 and organic acid levels were analysed for significant differences by analysis of variance (ANOVA)
198 with the JMP 8 (SAS Institute Inc, NC, USA) statistical package. Statistical significance was
199 defined as $P<0.05$; when the analysis was statistically significant, a Tukey test for separation of
200 means was performed.

201 **3. Results**

202 **3.1. *P. digitatum pg1* and *pg2* gene knockout mutants**

203 We followed a gene deletion approach to study the role of the two major PGs from *P. digitatum*.
204 The promoter and terminator regions of the genes *pg1* and *g2* were PCR-amplified and cloned into
205 the binary plasmid pRF-HU2 flanking the hygromycin B resistance cassette, originating plasmids
206 pRFDPG1 and pRFDPG2, respectively. After *A. tumefaciens*-mediated transformation of *P.*
207 *digitatum* Pd1, 50 and 96 transformants of *pg1* and *pg2*, respectively, were screened by PCR to
208 detect the presence of the hygromcycin B resistance marker with primers HMBF1 and HMBR1
209 (Table1). All transformants were positive for the amplification of the expected 801 bp amplicon
210 (Fig. 1B and 2B). We analysed the occurrence of double homologous recombination at the *pg1* and
211 *pg2* loci using primer pairs pg1-F7/pg1-R7 and pg2-F7/pg2-R7 for *pg1* and *pg2*, respectively. Eight
212 *pg1* transformants and 10 *pg2* transformants showed a single amplicon of the expected size, 3.4 kb
213 for *pg1* and 3.0 kb for *pg2* (Fig. 1B and 2B). The absence of the wild type band (1.9 kb and 1.7 kb
214 for *pg1* and *pg2*, respectively) in these transformants is indicative of gene replacement, thus
215 originating Δ*pg1* and Δ*pg2* null mutants. The remaining ectopic transformants showed two bands,
216 the lower band corresponding to the original locus and the upper one corresponding to the T-DNA.

Further confirmation of gene replacement in Δ*pg1* and Δ*pg2* deletants was observed by the lack of amplification with gene-specific primers pg1-F8/pg1-R8 and pg2-F8/pg2-R8 for *pg1* and *pg2*, respectively, (Fig. 1B and 2B). Amplification of a 700 or 650 bp band, for *pg1* and *pg2* respectively, with these primers was only observed in the wild type Pd1 strain and the ectopic transformants. The disrupted Δ*pg1* allele only contains a fragment of the *pg1* gene encompassing the first 75 aa of the 367 aa of the PG1 protein, whereas the disrupted Δ*pg1* allele only contains the first 85 aa of the 378 aa. Hence, both disrupted Δ*pg* mutants lack a functional gene.

Four deletant and two ectopic transformants for each gene were selected for determination of the number of T-DNA copies integrated in the genome by quantitative PCR using the wild type Pd1 strain as a control and the β-tubulin gene as the reference. All *pg1* transformants contained only one copy of T-DNA. However, only two Δ*pg2* deletants and one ectopic transformant contained one copy of the T-DNA. The other two Δ*pg2* deletants contained two T-DNA copies and the second ectopic transformant contained three copies of the T-DNA integrated in the genome. Two deletants and one ectopic transformant for each gene containing a single T-DNA integrated in the genome were selected for further analysis. As shown in Fig. 1C and 2C growth and sporulation of selected deletant and ectopic transformants did not differ from the wild type strain.

3.2. Development of green mould caused by *P. digitatum pg* transformants

Fruit maturity stage was characterized by measuring colour index, firmness, soluble solids and acidity. Results for colour index and firmness on 'Valencia' oranges were 3.0 and 3.97 mm, respectively. Results for soluble solids and acidity were 11.0 % and 0.9 g L^{-1} citric acid, respectively. In comparison to previous studies on 'Valencia' oranges (Vilanova et al., 2012,

238 Vilanova et al., 2013), our quality results indicated that oranges used in this study were at
239 commercial maturity stage.

240 To evaluate the effect of both genes in virulence, oranges were inoculated with the wild type *P.*
241 *digitatum* Pd1 and two Δ*pg* mutants and one ectopic transformant for each gene. Our results
242 showed that deletion of either *pg1* or *pg2* did not affect the decay incidence (Fig. 3A), but the
243 lesion diameter in oranges inoculated with Δ*pg1* (ΔPG1-8 and ΔPG1-10) and Δ*pg2* (ΔPG2-0 and
244 ΔPG2-13) was smaller than in those fruits inoculated the wild type strain after 4 days of inoculation
245 (Fig. 3B). The average reduction in decay severity of the ΔPG1-8 and ΔPG1-10 was 31 and 33 %,
246 respectively compared to the wild type strain, however no significant differences were found
247 between its respective ectopic (EPG1-5) and the wild type strain. The average reduction in decay
248 severity of the ΔPG2-0 and ΔPG2-13 was 47 and 51 %, respectively compared to the wild type
249 strain. In addition, its respective ectopic mutant (EPG2-5) also showed lower lesion diameter than
250 the wild type strain. In pathogenicity assays conducted with a lower inoculum dose (10^4 conidia
251 mL^{-1} instead of 10^6 conidia mL^{-1}) we observed the same results. Although there were no differences
252 in disease incidence between the Δ*pg* deletants and the wild type strain, decay severity was lower
253 in fruits inoculated with the Δ*pg2* deletants (results not shown).

254 **3.3. Changes in pH, firmness and polygalacturonase activity induced by *P. digitatum* mutants**
255 **in orange decay**

256 The behaviour showed by the different mutants and the wild type strain in relation to pH, firmness
257 and PG activity was evaluated at four days after inoculation (Fig. 4).

258 *P. digitatum* wild type strain decreased orange peel pH from approximately 4.8 in control tissue
259 (oranges inoculated with water) to approximately 3.0, representing around 38 % reduction (Fig.

4A). No significant differences were observed among the pH of the Δ*pg1* ectopic and null mutants (EPG1-5, ΔPG1-8 and ΔPG1-10) and the pH of the wild type strain. However, the pH value in fruits inoculated with the ΔPG2-0 and ΔPG2-13 deletants (3.2 and 3.1, respectively) was slightly higher than that measured in the wild type strain (3.0). Moreover, no significant differences were observed between the pH of EPG2-5 and the wild type strain.

Concerning firmness values, the *P. digitatum* wild type strain decreased the firmness of orange peel from approximately 6.32 N in control tissue to approximately 1.46 N, representing a reduction around 76 % (Fig. 4B). No significant differences in firmness were observed among the *pg1* ectopic and null mutants (EPG1-5, ΔPG1-8 and ΔPG1-10) and the wild type strain. However, firmness of the ΔPG2-0 and ΔPG2-13 deletants (3.17 and 3.87 N, respectively) was markedly higher than that measured in the wild type strain (1.46 N). No significant differences in firmness were observed between the ectopic EPG2-5 and the wild type strain.

Large differences were found in PG activity levels between control tissue and the wild-type strain, activity values being approximately 20-fold higher in the latter (0.92 vs. 18.4 U mg^{-1} protein, respectively) (Fig. 4C). No significant differences were observed among the PG activity of the *pg1* ectopic (EPG1-5) and ΔPG1-8 null mutant and the wild type strain. However ΔPG1-10 showed lower PG activity (12.3 U mg^{-1} protein) than the wild type strain (18.4 U mg^{-1} protein). The lowest PG activity was found in Δ*pg2* null mutants ΔPG2-0 and ΔPG2-13 (10.3 and 11.2 U mg^{-1} protein, respectively). Activity levels in both Δ*pg2* null mutants were significantly lower in comparison with their respective ectopic mutant (EPG2-5), which showed however similar levels in comparison with the wild-type strain.

3.4. Changes in organic acids induced by *P. digitatum* mutants in orange decay

Malic, ascorbic, oxalic, citric and fumaric acid level caused by the different mutants and the wild type strain was quantified after four days of inoculation. In general, no significant differences were observed among wild type strain and PG transformants (data not shown). Ascorbic acid level showed significant differences among control tissue (1.597 mg g^{-1} DW) and that mutants with less lesion diameter: *pg2* ectopic and null mutants also showed lower ascorbic acid levels (0.766, 0.866 and 0.516 mg g^{-1} DW, respectively) than the wild type strain. Moreover, the lowest ascorbic acid level was detected in ΔPG2-13 (0.516 mg g^{-1} DW).

Gluconic and galacturonic acid level caused by the different mutants and the wild type strain was quantified after four days of inoculation (Fig. 5) Gluconic acid level detected in control tissue was 18.666 mg g^{-1} DW (Fig. 5A). However, no significant differences were observed among control tissue and *pg1* and *pg2* ectopic and null mutants and the wild type strain (in a range of 17.791 to 20.408 mg g^{-1} DW).

P. digitatum wild type strain increased galacturonic acid level from approximately 7.520 mg g^{-1} DW in control tissue to approximately 108.087 mg g^{-1} DW, being approximately 14-fold higher in the wild type (Fig. 5B). No significant differences were observed among the *pg1* ectopic and null mutants (115.187, 118.718 and 110.794 mg g^{-1} DW, respectively) and the wild type strain (108.087 mg g^{-1} DW). However, the quantity observed in the ΔPG2-0 and ΔPG2-13 (55.645 and 54.727 mg g^{-1} DW, respectively) was lower than that measured in the wild type strain (108.087 mg g^{-1} DW) and the ectopic EPG2-5 mutant (135.541 mg g^{-1} DW).

4. Discussion

303 *P. digitatum* is a necrotrophic fungus that causes extensive maceration of the invaded citrus peel
304 tissue, presumably due to the action of pectinases secreted by the pathogen during the infection
305 process. A correlation between pectinases and orange peel maceration was observed in avirulent
306 strains of *P. digitatum* obtained by UV irradiation (Garber et al., 1965). These mutants only caused
307 necrotic lesions at the site of inoculation but did not cause rot. Pectinolytic activity was absent in
308 the necrotic tissue but abundant in macerated tissue from diseased fruits. The analysis of *P.*
309 *digitatum* pectinases obtained from culture filtrates showed that a secreted pectin lyase had
310 macerating activity on orange rind tissue (Bush and Codoner, 1968). However, the macerating
311 activity during lesion development was found to be associated with an exoPG purified from the
312 macerate peel of oranges infected with *P. digitatum* (Barmore and Brown, 1979).

313 We have previously identified in the genome of *P. digitatum* two PG encoding genes (*pg1*,
314 PDIP_64460, and *pg2*, PDIP_19910, respectively) and two genes encoding pectin lyases (*pnl1* and
315 *pnl2*, PDIP_08080 and PDIP_57790) that showed a strong up-regulation during the infection of
316 orange fruits (López-Pérez et al., 2015). *P. digitatum* mutants lacking either *pg2* (Zhang et al.,
317 2013b) or *pnl1* (López-Pérez et al., 2015) showed reduced virulence compared to their wild type
318 strains, although they were still able to develop infection on citrus fruit. In this work, we aimed to
319 compare the role in virulence and maceration capability of the two major PGs, PG1and PG2, from
320 *P. digitatum* by using a functional genomics approach. To avoid strain specific effects, we used
321 the same *P. digitatum* Pd1 strain for generating deletants of both *pg1* and *pg2*. This is the same
322 strain in which we have previously generated deletants lacking the pectin lyase PNL1 (López-Pérez
323 et al., 2015). Moreover, the genome sequence of this strain is the species' reference genome at the
324 NCBI's RefSeq database.

Binary T-DNA plasmids containing the upstream and downstream regions of both genes flanking a hygromycin B resistance cassette were constructed and introduced into *P. digitatum* via *Agrobacterium*-mediated transformation. Two independent deletants and one ectopic transformant containing just one copy of T-DNA for each gene were selected. All of them grew and sporulated as the wild type strain on PDA medium (Figure 1 and 2). Pathogenicity assays showed that both Δ*pg1* and Δ*pg2* deletants had the same disease incidence on oranges as the wild type strain or the ectopic transformants. However, disease severity progressed more slowly in the Δ*pg* deletants, specially in the two Δ*pg2* deletants, which showed an average reduction around 50% in lesion diameter with respect to the wild type strain. A similar reduction in disease severity was already observed in a Δ*pg2* deletant obtained in a different *P. digitatum* strain (Zhang et al., 2013b). Previous works conducted in *Botrytis cinerea*-infected tomatoes by Kars et al. (2005) showed that mutants in the *Bcpg2* gene had a >50 % reduction in virulence, meanwhile strains with a mutation in the *Bcpg1* gene only reduced its virulence by 25 % (ten Have et al., 1998). The role of both *P. digitatum* PGs to disassemble the orange cell wall seems to be critical for the full virulence of *P. digitatum*; however, the disruption of one pectinase gene appears not to be enough to fully reduce the virulence of this pathogen, probably due to the presence of multiple pectinases in the genome of *P. digitatum* (Marcet-Houben et al., 2012). The presence and abundance of pectins into the wall matrix is considered to regulate the wall extensibility, and different pectin domains crosslink to each other via calcium and boron bonds. Additional cell wall-related enzymes such as pectin methylesterases can modify these connections, and therefore increase the susceptibility of pectins to depolymerisation by PGs and pectate lyases within the wall (Caffall and Mohnen, 2009). Furthermore, the accessibility of these enzymes to their pectin-backbone substrate is modulated by

347 the activity of cell wall hydrolases acting on galactosyl- and arabinosyl-rich pectin side-chains,
348 which thus regulate cell wall porosity (Goulao and Oliveira, 2008).

349 We noticed that the lesions in fruits inoculated with both Δ*pg2* deletants were not as soft as those
350 observed in fruits inoculated with the wild type strain or any other transformants. Firmness values
351 of Δ*pg2* deletants were significant higher than those obtained with the wild type and the other
352 transformants (Fig 3B). This prompted us to analyse in more detail the characteristics of the
353 macerated tissue in the lesions originated by the different strains. PG activity in the Δ*pg2* deletants
354 showed a tendency to decrease in relation to wild type and the other transformants and this was
355 clearly related with higher decayed tissue firmness and lower galacturonic acid production. The
356 accumulation of galacturonic acid is a consequence of the complete pectin degradation by PG
357 activity as reported Barmore and Brown (1979). In the case of *B. cinerea*, up to 13 endoPG
358 isozymes have been described during the progress of the infection (van der Cruyssen et al., 1994).
359 All BcPGs resulted true endopolygalacturonases, however, they showed different modes of action.
360 PGA hydrolysis by PG1, PG2 and PG4 produced an accumulation of oligomers with DP < 7.
361 However, PGA hydrolysis by PG3 and PG6 produced an accumulation of monomers and dimmers
362 (Kars et al. 2005).

363 It is known that the timing and degree of *in planta* gene expression of the endoPG family differs
364 depending on the host tissue, the degree of fruit ripening, the infection stage and the temperature.
365 Besides pathogen PG activities, the peel also contains other cell wall degrading enzymes that
366 contribute to softening of the tissue. Wubben et al. (2000) attributed the different expression
367 patterns to four mechanisms: basal expression, induction by pectic monomers, repression of the
368 glucose and ambient pH modulation. In our study, the deletion of *pg2* resulted in decayed tissue
369 with higher firmness, lower PG activity and a 2-fold decrease in galacturonic acid level in relation

370 to the lesions originated by the wild type strain. These results demonstrated that Δ*pg2* strains had
371 a reduced capacity for pectin decomposition and, hence, less amount of galacturonic acid was
372 observed, and less nutrients were available for the fungus development. Restriction of growth may
373 not be solely due to restriction of nutrients but also by physical limitation of hyphal growth through
374 cells or more importantly between cells and air space. The monosaccharide D-galacturonic acid
375 seems an important component for the nutrition of *P. digitatum* because the Δ*pg2* transformants
376 showed less infection capacity, determined as the lesion diameter, which correlates with the
377 production of lower amounts of galacturonic acid in the orange peel, indicating that the gene *pg2*
378 is a virulence factor. Taking into account that the albedo of citrus fruit is very rich in pectin, the
379 capacity to degrade pectin by *P. digitatum* strain is critical to achieve a successful colonization of
380 the host. Also in *P. digitatum*, Zhang et al. (2013b) found that one endoPG gene (*Pdpg2*) and one
381 pectin lyase gene (*Pdpnl1*) were upregulated during citrus fruit infection in the wild type while not
382 in the *P. digitatum* Δ*pacC* mutant. The PacC transcription factor is the terminal component of the
383 pH signalling pathway. These authors hypothesize that PacC regulates the expression of some
384 genes that are required for the degradation of pectin in the citrus peel, such as polygalacturonases
385 and pectin lyases.

386 The significant pH decrease observed in orange tissue infected by the different *P. digitatum* strains
387 agrees with previous reports by other authors (Prusky et al., 2004; Zhang et al., 2013b; Vilanova
388 et al., 2014). From our results, the optima pH required by both PG from *P. digitatum* was at least
389 between 3.0-3.2. Maceration tissue was clearly correlated with a decrease in pH, independently of
390 the lesion diameter. No significant differences among wild type and both Δ*pg1* were observed, but
391 there was a significant difference with the pH of the tissue infected by both Δ*pg2* deletants. The
392 results obtained in this work confirm previous work that showed that galacturonic acid was not

393 responsible for the pH decrease observed in infected orange tissue (Vilanova et al., 2014); however,
394 the difference in pH level between Δ*pg1* and Δ*pg2* (around 0.2 pH units) could be related to
395 galacturonic acid content.

396 Different approaches to evaluate the role of CWDEs in pathogenesis have been conducted in other
397 pathogens such as *Alternaria citris* (Akimutsi et al., 2004). They showed that the PG is essential
398 for degradation of the plant cell wall components, mainly pectin, and for citrus fruit colonization
399 and pathogenesis.

400 In conclusion, by obtaining gene knockout mutants, we have shown that the two major PGs, PG1
401 and PG2, produced by *P. digitatum* during infection of citrus fruit are required for full virulence.
402 These two proteins seem to be dispensable to establish infection but they play a role in the
403 colonization of the orange peel. Moreover, although these two proteins are endopolygalacturonases
404 they show distinct enzymatic properties *in vivo*. Hence, PG2 plays a major role than PG1 in tissue
405 softening, pH reduction and galacturonic acid production.

406

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Figure legends

Fig. 1. Analysis of *Penicillium digitatum pg1* transformants. (A) Diagram of the wild-type locus and the *pg1* replacement with the Hyg^R selectable marker from pRFDPG1 by homologous recombination to generate the Δ*pg1* mutant. Primers used in the construction of plasmid pRFDPG1

516 and those used for the analysis of the transformants are shown. (B) Polymerase chain reaction
517 (PCR) analysis of the wild-type Pd1 strain, an ectopic (Epg1-5) and two knockout (Δpg1-8 and
518 Δpg1-10) transformants. (C) Growth of the wild type *P. digitatum* Pd1, an ectopic (Epg1-5) and
519 two knockout Δ*pg1* mutants (Δpg1-8 and Δpg1-10) after 7 days of incubation at 24 ºC on PDA
520 medium.

521 **Fig. 2.** Analysis of *Penicillium digitatum pg2* transformants. (A) Diagram of the wild-type locus
522 and the *pg2* replacement with the Hyg^R selectable marker from pRFDPG2 by homologous
523 recombination to generate the Δ*pg2* mutant. Primers used in the construction of plasmid pRFDPG2
524 and those used for the analysis of the transformants are shown. (B) Polymerase chain reaction
525 (PCR) analysis of the wild-type Pd1 strain, an ectopic (Epg2-5) and two knockout (Δpg2-0 and
526 Δpg2-13) transformants. (C) Growth of the wild type *P. digitatum* Pd1, an ectopic (Epg2-5) and
527 two knockout Δ*pg2* mutants (Δpg2-0 and Δpg2-13) after 7 days of incubation at 24 ºC on PDA
528 medium.

529 **Fig. 3.** Disease incidence (A) and lesion diameter (B) in 'Valencia' oranges inoculated with
530 different *P. digitatum pg1* transformants at 10^6 conidia mL^{-1} and stored at 20 ºC and 85 % RH for
531 4 days. Wild type *P. digitatum* (Pd1), two ectopic mutants (EPG) and four knockout mutants (ΔPG)
532 were analyzed. Each column represents the mean of four replicates and each replicate consisted of
533 five fruits with two wounds per fruit. Samples with different letters are significantly different
534 according to Tukey test (P<0.05).

535 **Fig. 4.** pH (A), firmness (B) and polygalacturonase (PG) activity in 'Valencia' oranges inoculated
536 with different *P. digitatum pg1* transformants at 10^6 conidia mL^{-1} and water as control treatment
537 and stored at 20 ºC and 85 % RH for 4 days. Wild type *P. digitatum* (Pd1), two ectopic mutants

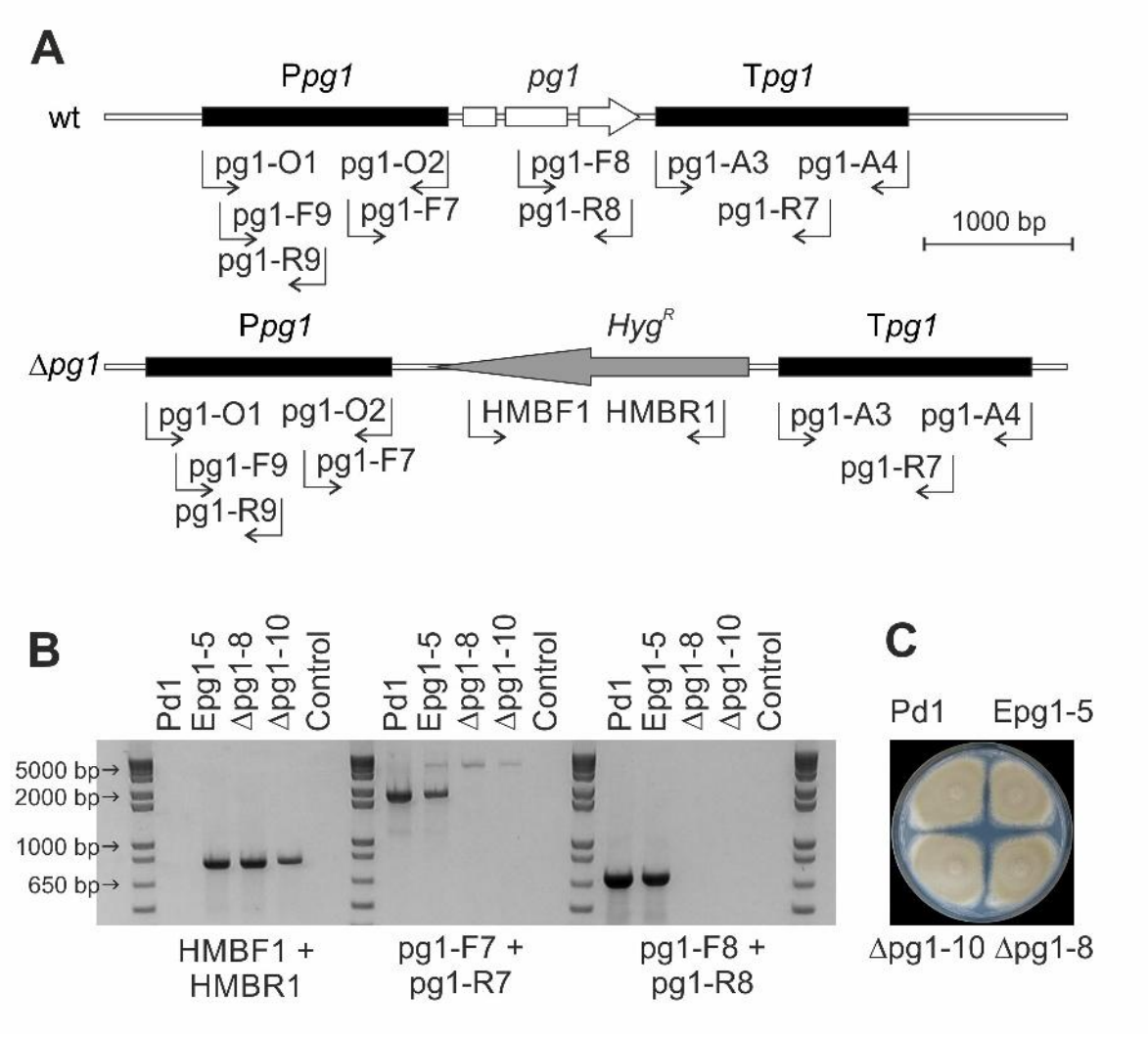
538 (EPG) and four knockout mutants (ΔPG) were analyzed. For pH, each column represented the
539 mean of four replicates and for firmness, each column represented the mean of two replicates. In
540 both cases, each replicate consisted of five fruits with two wounds per fruit. For polygalaturonase
541 (PG) activity, each column represented the mean of three replicates and each replicate consisted of
542 ten fruits with two wounds per fruit. Samples with different letters are significantly different
543 according to Tukey test (P<0.05).

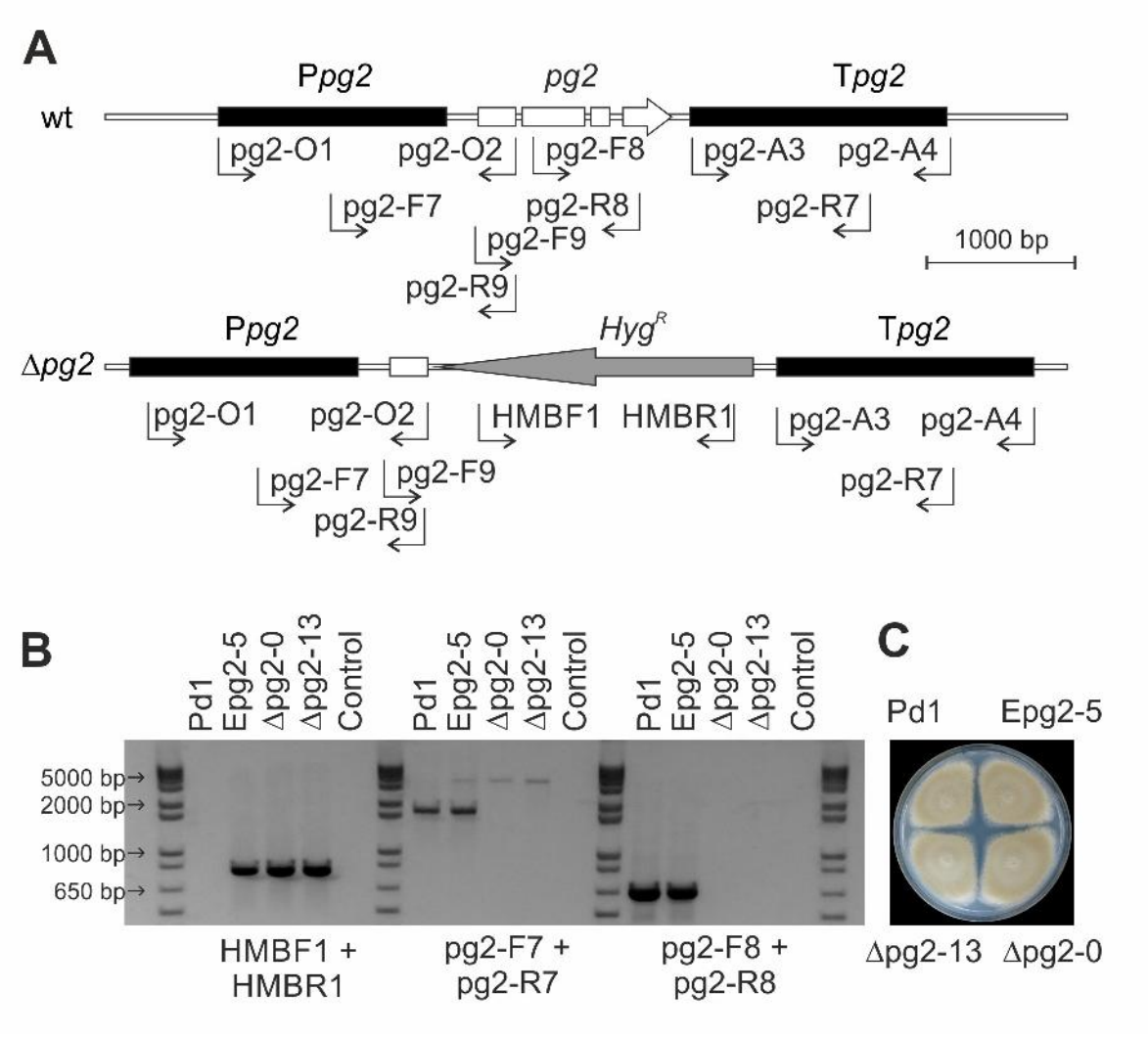
544 **Fig. 5.** Gluconic (A) and galacturonic (B) acid levels in 'Valencia' oranges inoculated with *P.*
545 *digitatum pg1* transformants at 10^6 conidia mL^{-1} and water as control treatment and stored at 20 ºC
546 and 85 % RH for 4 days. Wild type *P. digitatum* (Pd1), two ectopic mutants (EPG) and four
547 knockout mutants (ΔPG) were analyzed. Each column represents the mean of four replicates and
548 each replicate consisted of ten fruits with two wounds per fruit. Samples with different letters are
549 significantly different according to Tukey test (P<0.05).

550

Table 1. Primers used in this study

Name	Sequence (5' to 3')	Purpose
pg1-O1	GGTCTTAAUGCCCCACTGGTCGATCCTAACCTTCCA	Amplification of the upstream region of *pg1*
pg1-O2	GGCATTAAUTGGGGGTTGACGCTTGCATAACAGAGC	Amplification of the upstream region of *pg1*
pg1-A3	GGACTTAAUGCCAGCGATCAAATGGTGAACACCAAAC	Amplification of the downstream region of *pg1*
pg1-A4	GGGTTTAAUAAGCGTCTGCGTGGTGGTGTGCAGT	Amplification of the downstream region of *pg1*
pg2-O1	GGTCTTAAUTGCGTGGTCTGTGGGGTGGTCGTTT	Amplification of the upstream region of *pg2*
pg2-O2	GGCATTAAUTGGGTGCCGGTGTTCAATCCAGTCA	Amplification of the upstream region of *pg2*
pg2-A3	GGACTTAAUTTTGACTCCTTGCTGGCCGGGCTTG	Amplification of the downstream region of *pg2*
pg2-A4	GGGTTTAAUTCCGCTCGTGAACAGGAGCACGTTG	Amplification of the downstream region of *pg2*
RF-1	AAATTTTGTGCTCACCGCCTGGAC	Analysis of plasmid constructs
RF-2	TCTCCTTGCATGCACCATTCCTTG	Analysis of plasmid constructs
RF-5	GTTTGCAGGGCCATAGAC	Analysis of plasmid constructs
RF-6	ACGCCAGGGTTTTCCCAGTC	Analysis of plasmid constructs
HMBF1	CTGTCGAGAAGTTTCTGATCG	Amplification of the hygromycin B resistance marker
HMBR1	CTGATAGAGTTGGTCAAGACC	Amplification of the hygromycin B resistance marker
pg1-F7	AAGCTCGATGGAATAGCTT	Detection of double homologous recombination at the *pg1* locus
pg1-R7	CCCAGTAAAAGGACATGC	Detection of double homologous recombination at the *pg1* locus
pg1-F8	AAAGAAGAAGCCCAAGTTCT	Detection of *pg1*
pg1-R8	AGCTACCGTTACCGCAGAGA	Detection of *pg1*
pg2-F7	ATGCTATTGGTTCTTTCCTC	Detection of double homologous recombination at the *pg2* locus
pg2-R7	TCCCTCCGTAAACTAAACAA	Detection of double homologous recombination at the *pg2* locus
pg2-F8	TCGATGGCGCTAAGGAGCTTACT	Detection of *pg2*
pg2-R8	CTCGGCACACAGAATGTA	Detection of *pg2*
pg1-F9	CGGACGGAGTAGATCTCACAACT	Determination of T-DNA copy number in *pg1* transformants
pg1-R9	CCTGCGCTAACATCCTCATGAAAC	Determination of T-DNA copy number in *pg1* transformants
pg2-F9	CCTCGTGGTGCTTGTACCTTCTC	Determination of T-DNA copy number in *pg2* transformants
pg2-R9	TCAGGGTAATGGTCGAGCAAGC	Determination of T-DNA copy number in *pg2* transformants
betatubPDIG1	CGATGGCGATGGACAGTAAGTTT	Determination of T-DNA copy number in *pg1* and *pg2* transformants
betatubPDIG2	TTGGTTCGTGGTCGTTGTACTCA	Determination of T-DNA copy number in *pg1* and *pg2* transformants


A
Ppg1
pg1
Tpg1
wt
pg1-O1
pg1-O2
pg1-F8
pg1-A3
pg1-A4
pg1-F9
pg1-F7
pg1-R8
pg1-R7
1000 bp
pg1-R9
Ppg1
HygR
Tpg1
Δpg1
pg1-O1
pg1-O2
HMBF1
HMBR1
pg1-A3
pg1-A4
pg1-F9
pg1-F7
pg1-R7
pg1-R9
B
Pd1
Epg1-5
Δpg1-8
Δpg1-10
Control
5000 bp→
2000 bp→
1000 bp→
650 bp→
HMBF1 + HMBR1
pg1-F7 + pg1-R7
pg1-F8 + pg1-R8
C
Pd1
Epg1-5
Δpg1-10
Δpg1-8


A
wt
Ppg2
pg2
Tpg2
pg2-O1
pg2-O2
pg2-F8
pg2-A3
pg2-A4
pg2-F7
pg2-R8
pg2-R7
pg2-F9
pg2-R9
1000 bp
Δpg2
Ppg2
HygR
Tpg2
pg2-O1
pg2-O2
HMBF1
HMBR1
pg2-A3
pg2-A4
pg2-F7
pg2-F9
pg2-R7
pg2-R9
B
Pd1
Epg2-5
Δpg2-0
Δpg2-13
Control
5000 bp
2000 bp
1000 bp
650 bp
HMBF1 + HMBR1
pg2-F7 + pg2-R7
pg2-F8 + pg2-R8
C
Pd1
Epg2-5
Δpg2-13
Δpg2-0

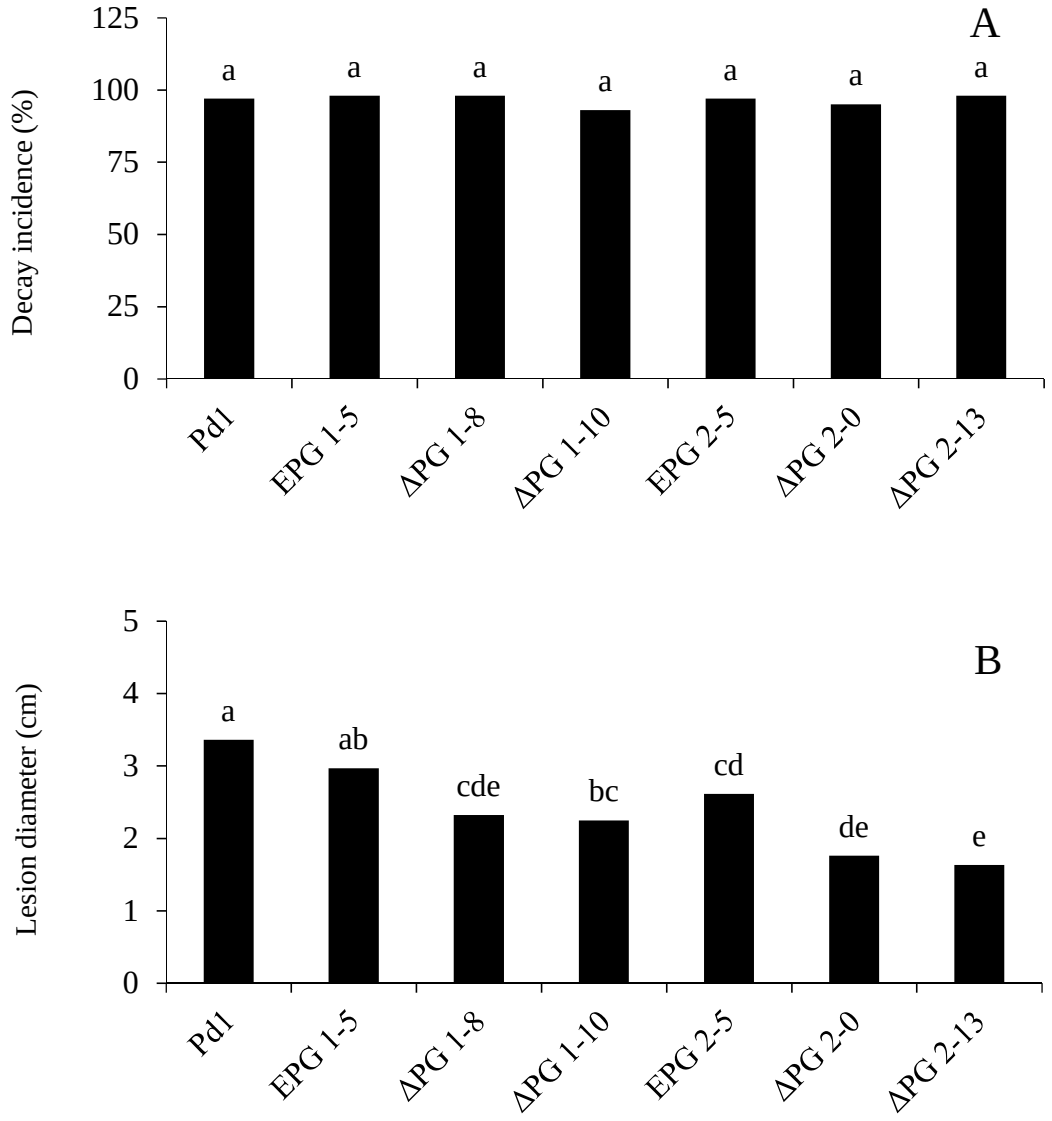

A
Decay incidence (%)
125
100
75
50
25
0
a
a
a
a
a
a
a
Pd1
EPG 1-5
ΔPG 1-8
ΔPG 1-10
EPG 2-5
ΔPG 2-0
ΔPG 2-13
B
Lesion diameter (cm)
5
4
3
2
1
0
a
ab
cde
bc
cd
de
e
Pd1
EPG 1-5
ΔPG 1-8
ΔPG 1-10
EPG 2-5
ΔPG 2-0
ΔPG 2-13

Fig. 3

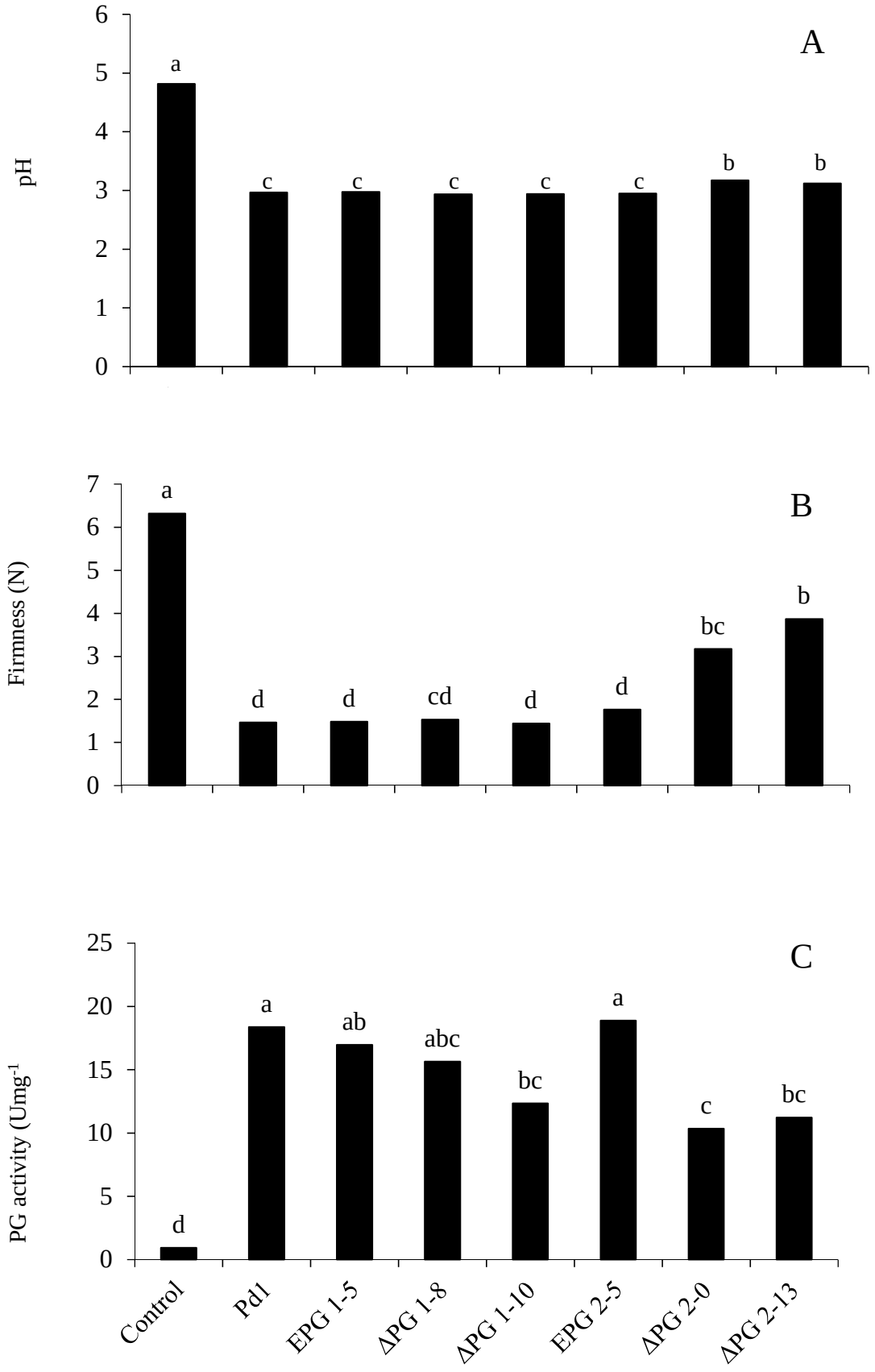

A
pH
6
5
4
3
2
1
0
a
c
c
c
c
c
b
b
B
Firmness (N)
7
6
5
4
3
2
1
0
a
d
d
cd
d
d
bc
b
C
PG activity (Umg-1
25
20
15
10
5
0
d
a
ab
abc
bc
a
c
bc
Control
Pd1
EPG 1-5
ΔPG 1-8
ΔPG 1-10
EPG 2-5
ΔPG 2-0
ΔPG 2-13

Fig 4

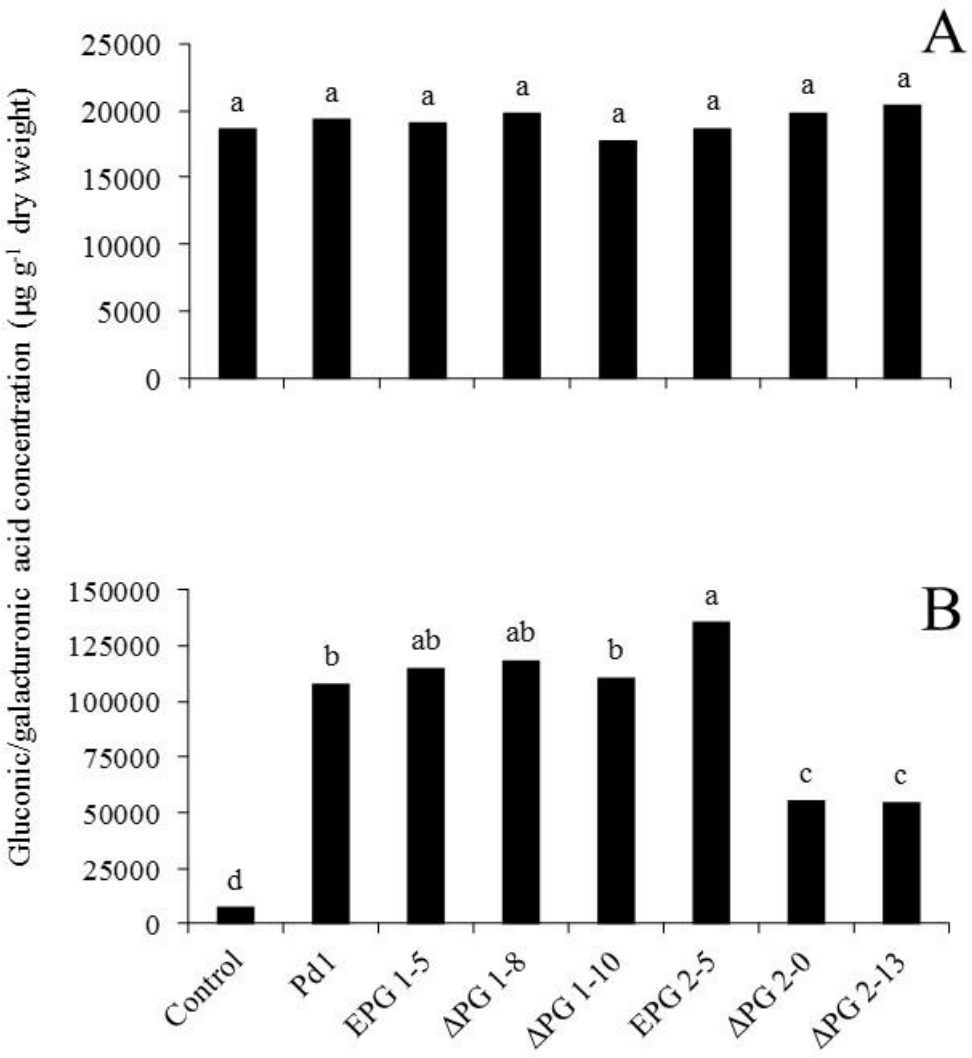


Fig. 5

Imagen en jpg